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Applicant: Tuszyński, Mark H.
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NEURODEGENERATIVE
DISEASE OF THE BRAIN
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DECLARATION OF MARK H. TUSZYNSKI, M.D.

I, Dr. Mark H. Tuszyński, declare as follows:

1. I am a Professor of Neurosciences and Director of the Center for Neural Repair at the University of California, San Diego (UCSD). I practice medicine as an attending neurologist at the UCSD and VA Medical Centers in La Jolla, California, and as the lead neurologist in ongoing clinical trials for treatment of Alzheimer's Disease (AD) using gene therapy in humans. I am also an inventor of the invention claimed in this US Patent Application Serial No. 09/060,543 (methods for *in vivo* gene therapy of neurodegenerative conditions), and in U.S. Patent No. 6,167,888 (claiming methods for *ex vivo* gene therapy of neurodegenerative conditions).

Background

2. The *in vivo* gene therapy method which is the subject of the present patent application has been tested in non-human primates and rodents, in preparation for human clinical studies to begin later this year. The *ex vivo* method which is the subject of the '888 patent is already being used in clinical trials, for treatment of AD.

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3. Effective gene therapy generally requires that a therapeutic gene product be expressed in target cells at levels, and for a duration, sufficient to provide a therapeutic benefit, without harmful immune responses to the delivery vehicle or gene product. In gene therapy of neurodegenerative disease, the goal is not gene replacement, nor is it permanently active expression of a therapeutic neurotrophin in the brain. Instead, relatively transient expression of neurotrophins can be sufficient to stimulate neuronal growth to a therapeutically significant degree. Thus, the eventual loss of expression (whether through immune processes, loss of promoter activity or other causes) is of less concern in the invention than in other therapeutic contexts.
4. Further, in the relatively immune-protected environment of the brain, immune responses to exogenous material are typically muted. In this respect, our findings are consistent with recent evidence indicating that viral vectors elicit only transient inflammation, if any, when *inserted directly* into brain parenchyma (as is done in the invention), versus entry by other means of delivery into the brain (e.g., infusion).

Overview of Data

5. We have used adeno-associated viral vectors to deliver neurotrophins by *in vivo* and *ex vivo* methods into the brains of humans, and have tested lentiviral vectors to the same ends in non-human primates. With particular respect to the *in vivo* work that is the subject of this patent application, we utilized aged monkeys which model neurodegeneration experienced in Parkinson's Disease (PD) or Alzheimer's Disease (AD). Animals modeling PD were treated with lenti-glial derived neurotrophic factor (GDNF), with lenti- β -galactosidase (GAL) serving as a control. Animals modeling AD were treated with lenti-nerve growth factor (NGF), with lenti-enhanced green fluorescent protein (eGFP) serving as a control.
6. In these experiments, we achieved expression of neurotrophin in targeted neurons at a level, and for a duration, believed to be sufficient for our therapeutic goals. Indeed, as many as >90% of targeted neurons can be efficiently transduced using the method of the

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invention, a visible demonstration of which is provided in enclosed Declaration Figure 1 (Dec.Fig. 1). In the Figure, GFP labeled treated neurons (using antibodies to glial filament protein (GFAP) or NeuN neuronal nuclear protein) show bright green if infected by the vectors employed in our experiments (a few such neurons are highlighted with arrows in each of the three photographs included in Dec. Fig. 1). Further, neither significant anti-vector immune responses nor cytotoxicity have been experienced in treated animals or people.

7. Representative data in these respects (not including confidential data from the human clinical trials) is provided in the present patent application, at page 7 and page 20 (Example II); in co-pending U.S. patent application 10/032,952; and in this Declaration.

PD Animal Models: Protocol, Expression and Lack of Immune Response

8. In the PD animal models, treatment with GDNF according to the invention stimulated increased production of dopamine in the brain (a critical protein for mitigation of PD), increased neuronal density around treated areas and, most significantly, resulted in improved motor function as measured by several criteria in treated animals.
9. More specifically, each monkey received six stereotaxic injections of lenti- Gal or lenti- GDNF bilaterally into the caudate nucleus, putamen, and substantia nigra. Injections were made into the head of the caudate nucleus (10 μ l), body of the caudate nucleus (5 μ l), anterior putamen (10 μ l), commissural putamen (10 μ l), postcommissural putamen (5 μ l), and substantia nigra (5 μ l). Injections were made through a 10- μ l Hamilton syringe connected to a pump at a rate of 0.5 μ l/min.
10. The left side was injected 6 weeks before the right. During the first surgical session, there was a technical failure with the virus aggregating in the needle, which prevented its injection into the brain. Thus, the left side served as an additional control for the right side. Postmortem, all GDNF injections were localized to the caudate nucleus, putamen, and supranigral regions, as revealed by standard staining procedures.

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11. Expression levels of the neurotrophin were confirmed by *in vitro* measurement of immunoreactivity with antibody in post-mortem tissue sections. All aged monkeys receiving lenti-GDNF displayed robust GDNF immunoreactivity within the right striatum (Dec.Fig. 2A) and substantia nigra (Dec.Fig. 2C), indicating expression and secretion of the neurotrophin had occurred. No monkeys receiving lenti- β -Gal displayed specific GDNF immunoreactivity in the right striatum (Dec.Fig. 2B), but each did display robust expression of β -Gal. No immune staining for antibodies against the lentiviral vector itself was observed.
12. Sections from all monkeys were stained for CD45, CD3, and CD8 markers to assess the immune response after lentiviral vector injection. These antibodies are markers for activated microglia, T cells, and leukocytes including lymphocytes, monocytes, granulocytes, eosinophils, and thymocytes. Staining for these immune markers was weak, and often absent, in these animals. Mild staining for CD45 and CD8 was seen in two animals. Other monkeys displayed virtually no immunoreactivity even in sections containing needle tracts.
13. Expression of GDNF in treated animals persisted for a relatively long duration, sufficient to stimulate the neuronal growth sought in the invention. GDNF-containing fibers emanating from putaminal injection sites were seen coursing medially toward and into the globus pallidus (Dec.Fig. 2D). These staining patterns were clearly distinct from the injection site and respected the boundaries of the striatal target structures. In contrast, anterograde transport of β -Gal was not observed in lenti- Gal monkeys. This suggests that secreted GDNF, and not the virus per se, was anterogradely transported.

Neurotrophin-induced improvements in motor function and neuronal density
in PD animal models

14. In response to the therapy, dopamine levels in GDNF treated animals increased significantly. Aged monkeys underwent fluorodopa (FD) positron emission tomography (PET) before surgery and again just before being killed. Before treatment, all monkeys displayed symmetrical FD uptake in the caudate and putamen bilaterally (ratio: 1.02 ± 0.02). Three of four lenti-GDNF-treated monkeys displayed clear increases in FD uptake on the treated side. Within the striatum, lentiviral delivery of GDNF increased a number of markers of dopaminergic function. For example, relative to control animals, measurement of dopamine (DA) and homovanillic acid (HVA) revealed significant increases in the right caudate nucleus (140% DA, $P < 0.001$; 207% HVA, $P < 0.001$) and putamen (47.2% DA, $P < 0.05$; 128% HVA, $P < 0.01$) in lenti-GDNF-treated aged monkeys.
15. Neuronal density (growth) in treated animals also increased significantly. Stereological counts revealed an 85% increase in the number of TH-immunoreactive nigral neurons on the side receiving lentivirally delivered GDNF relative to control animals. Further, a 35% increase in neuronal volume was seen on the GDNF-treated side of the brain in lenti-GDNF-injected aged monkeys (lenti- Gal $10,707.5 \pm 333 \mu\text{m}^3$; lenti-GDNF $16,653.7 \pm 1240 \mu\text{m}^3$; $P < 0.001$).
16. Perhaps most significantly, an improvement in function was observed on treatment of animals using a second model of neurodegenerative changes similar to those occurring in PD. In these experiments, young adult monkeys received unilateral intracarotid injections of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) to induce extensive nigrostriatal degeneration, resulting in a behavioral syndrome characterized by robust motor deficits.

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17. 20 young adult rhesus were initially trained 3 days per week until asymptotic performance was achieved on a hand-reach task in which the time to pick up food treats out of recessed wells was measured. Each experimental day, monkeys received 10 trials per hand. Once per week, monkeys were also evaluated on a modified parkinsonian clinical rating scale (CRS). All monkeys then received an injection of 3 mg MPTP-HCl into the right carotid artery, initiating a parkinsonian state.
18. One week later, monkeys were evaluated on the CRS. Only monkeys displaying severe hemiparkinsonism with the classic crooked arm posture and dragging leg on the left side continued in the study ($n = 10$). Monkeys with this behavioral phenotype generally display the most severe lesions neuroanatomically and do not display spontaneous recovery behaviorally.
19. On the basis of CRS scores, monkeys were matched into two groups of five monkeys, which received on that day lenti- β -Gal or lenti-GDNF treatment. Using magnetic resonance imaging (MRI) guidance, all monkeys were given lentivirus injections into the caudate nucleus ($n = 2$), putamen ($n = 3$), and substantia nigra ($n = 1$) on the right side using the same injection parameters as in experiment 1. One week later, monkeys began retesting on the hand-reach task three times per week for 3 weeks per month.
20. For statistical analyses, the times for an individual week were combined into a single score. During the weeks of hand-reach testing, monkeys were also scored once per week on the CRS. Individuals blinded to the experimental treatment performed all behavioral assessments. Statistically significant differences between lenti-GDNF and lenti- β -Gal were discerned at post-treatment observations 6, 7, 8, and 9 (Kolmogorov-Smirnov test, $P < 0.04$ for each comparison).
21. Lenti-GDNF-treated animals also improved performance on the operant hand-reach task. Under the conditions before MPTP administration, animals in both groups performed this task with similar speed. However, after MPTP, all lenti- β -Gal-treated

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animals were severely impaired, with monkeys often not performing at all, or requiring more than the maximally allowed 30 s. In contrast, three of the four lenti-GDNF monkeys performed the task with the left hand at near-normal levels, whereas one lenti-GDNF-treated monkey was impaired and performed this task in a manner similar to the lenti- β -Gal-treated animals.

AD Animal Models: Protocol, Expression and Lack of Immune Response

22. In the AD animal models, treatment with NGF according to the invention increased neuronal density around treated areas and, most significantly, resulted in improved cognitive function as measured by several criteria in treated animals.
23. An animal model that mimics loss of cholinergic neurons in AD is transection of the fornix pathway connecting the septum from the hippocampus. Such transections cause retrograde degeneration of cholinergic and non-cholinergic cell bodies in the septal nucleus of rats and primates. 7 rats underwent fornix transaction, and an equal number of animals were utilized as controls.
24. Treatment protocols were followed, and responses to treatment evaluated, as described above with respect to the GDNF treated animals, except that treatment focused on the Ch4 region of the forebrain, a principal region of impairment in AD. Two injection sites were chosen per hemisphere. The vector dosage concentration (both of NGF expressing vectors for treatment, and GFP expressing vectors for controls) applied was 1.5 μ l/site, delivered over an interval of 5 minutes. Animals were sacrificed, and tissue analyzed, 3 weeks after treatment.

Neurotrophin-induced improvements in motor function and neuronal density
in AD animal models

25. As visually apparent from Dec.Fig. 1, neurons in the treated regions displayed vigorous GFP immunoreactivity in histology, indicating extensive transfection of target cells by the NGF expressing vectors. Neither cytotoxicity nor an immune response to the vector

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constructs (using CD3 and CD8 as markers) was observed (CD3: Dec.Fig. 3A-C; and CD8: Dec.Fig.3D).

26. As shown in Dec.Figs. 4-6, neuronal density (as measured by number [Dec.Figs. 4 and 6], and size [Dec. Figs. 5 and 6] of neurons) in treated animals increased significantly, approaching near normal levels (compare, e.g., Dec.Fig. 6C and B showing density measured in, respectively, young and NGF treated animals, versus aged control animals [Dec.Fig. 6A]).
27. Moreover, the growth was associated with increased expression of the p75 receptor. Expression of p75 is regulated by NGF, so that a loss of NGF signalling further reduces the amount of p75 present in the brain, which may contribute to a decline in retrograde NGF signalling.
28. There were significantly fewer p75-labeled neurons in Ch4 from untreated aged rats than in untreated young rats ($p < 0.01$). However, on treatment of the aged animals, the mean number of p75-labeled Ch4i neurons from NGF-grafted aged rats increased to a level not significantly different from numbers observed in the untreated young animals. See, Dec.Fig. 4.
29. The increases in neuronal density observed were of cholinergic neurons--those lost in AD. See, Dec.Fig. 7. These results demonstrate that cholinergic neurons in the rat brain can be rescued from loss through intraparenchymal delivery of NGF using the method of this invention.

Conclusion

30. From all of the foregoing results, as well as those outlined in the patent application, one can reasonably predict that the method of the invention may be successfully and effectively practiced in humans, to produce a therapeutic response to neurotrophins delivered for expression from vector constructs.

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31. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements are made with the knowledge that willful false statements are so made punishable by fine or imprisonment, or both, under Section 101 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

April 7, 2003

Dated

Mark H. Tuszynski

Dr. Mark H. Tuszynski

GFP labeling indicates that neurons are primarily infected with the *in vivo* vector

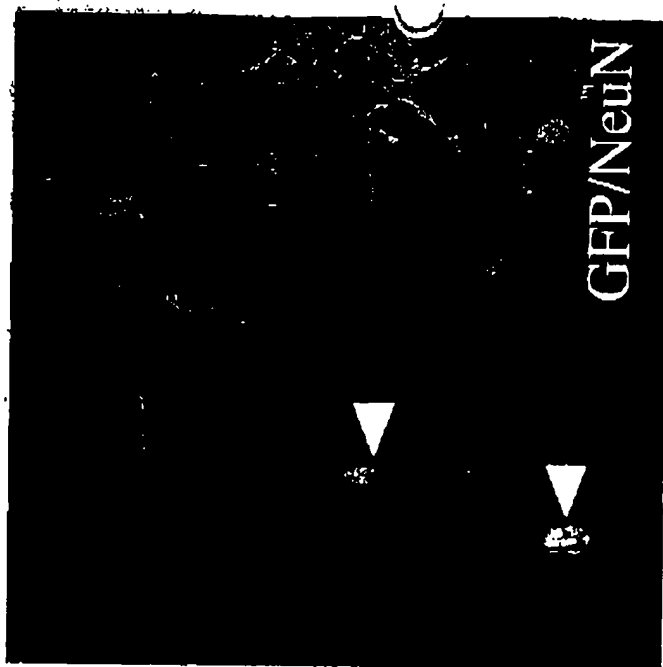
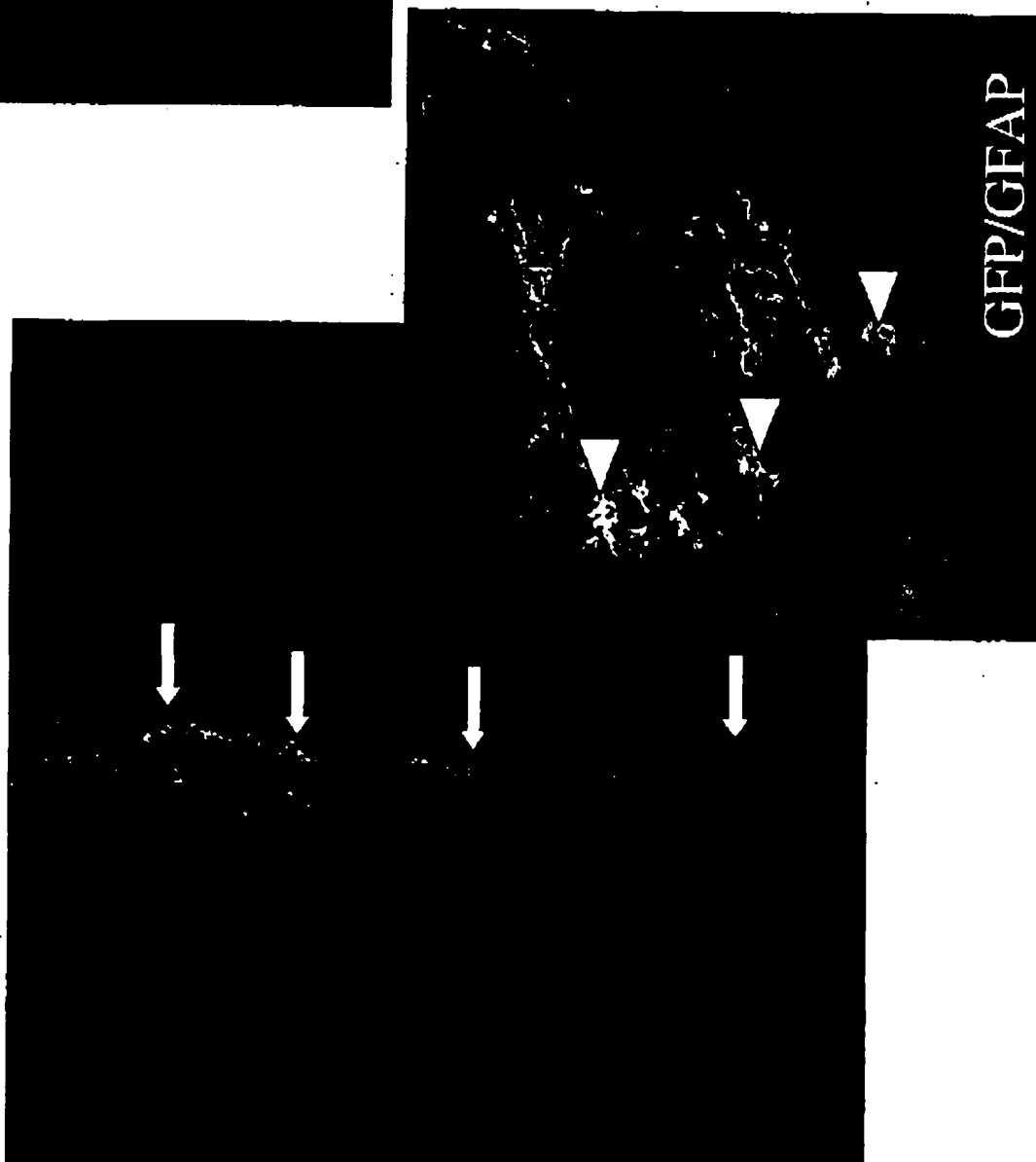


FIGURE 1



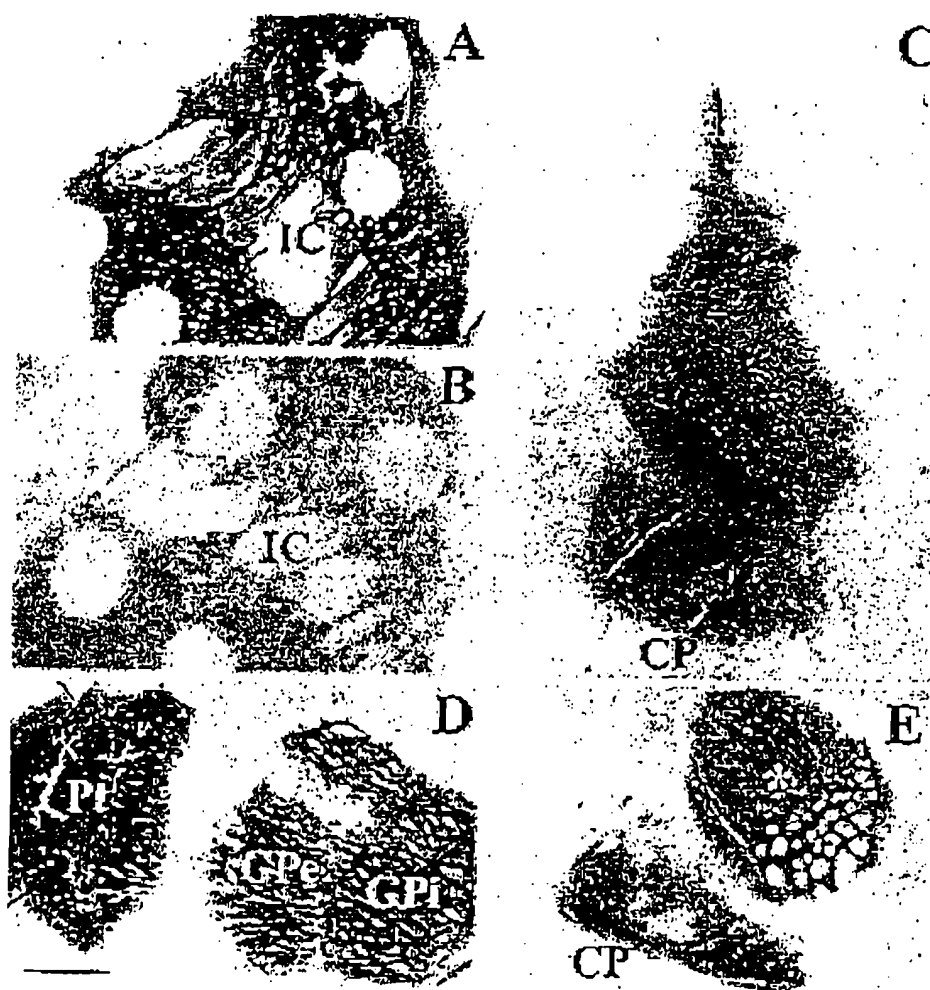
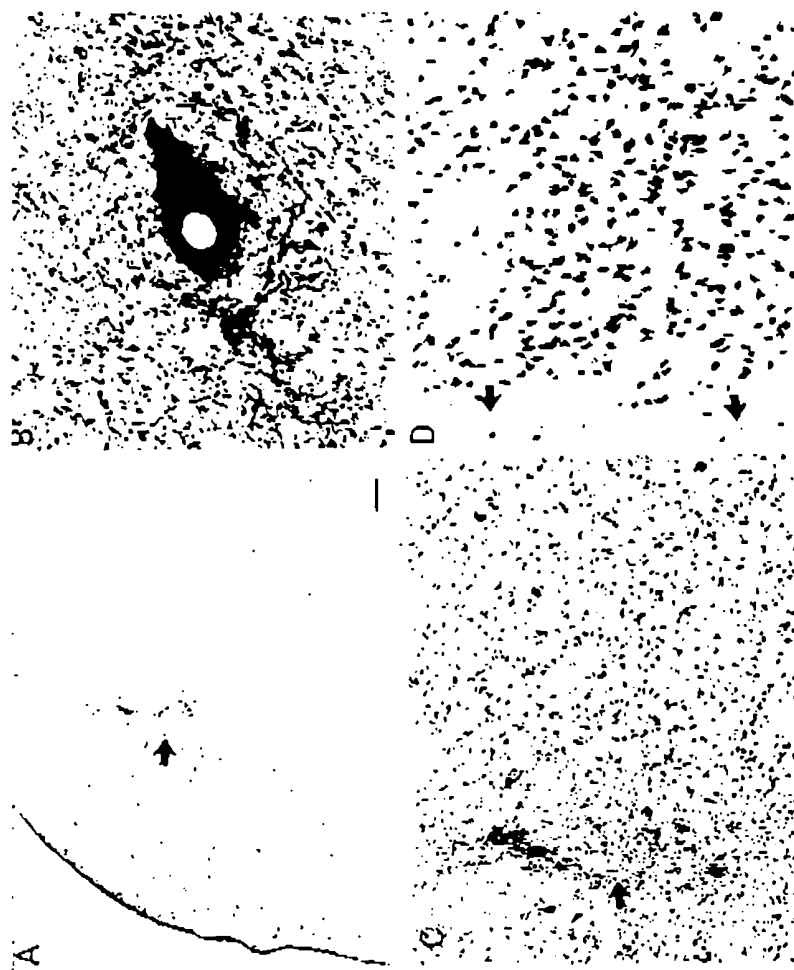


FIGURE 2

FIGURE 3

LENTIVIRAL GENE DELIVERY DOES NOT INDUCE IMMUNE RESPONSE OR CYTOTOXICITY



CD3 (A-C) and CD8 (D) labeling shows minimal or no inflammatory response to *in vivo* GDNF gene delivery using lentiviral vectors 3 months after injection into the rhesus monkey striatum

FIGURE 4

Number of p75-labeled Neurons in the Nucleus Basalis

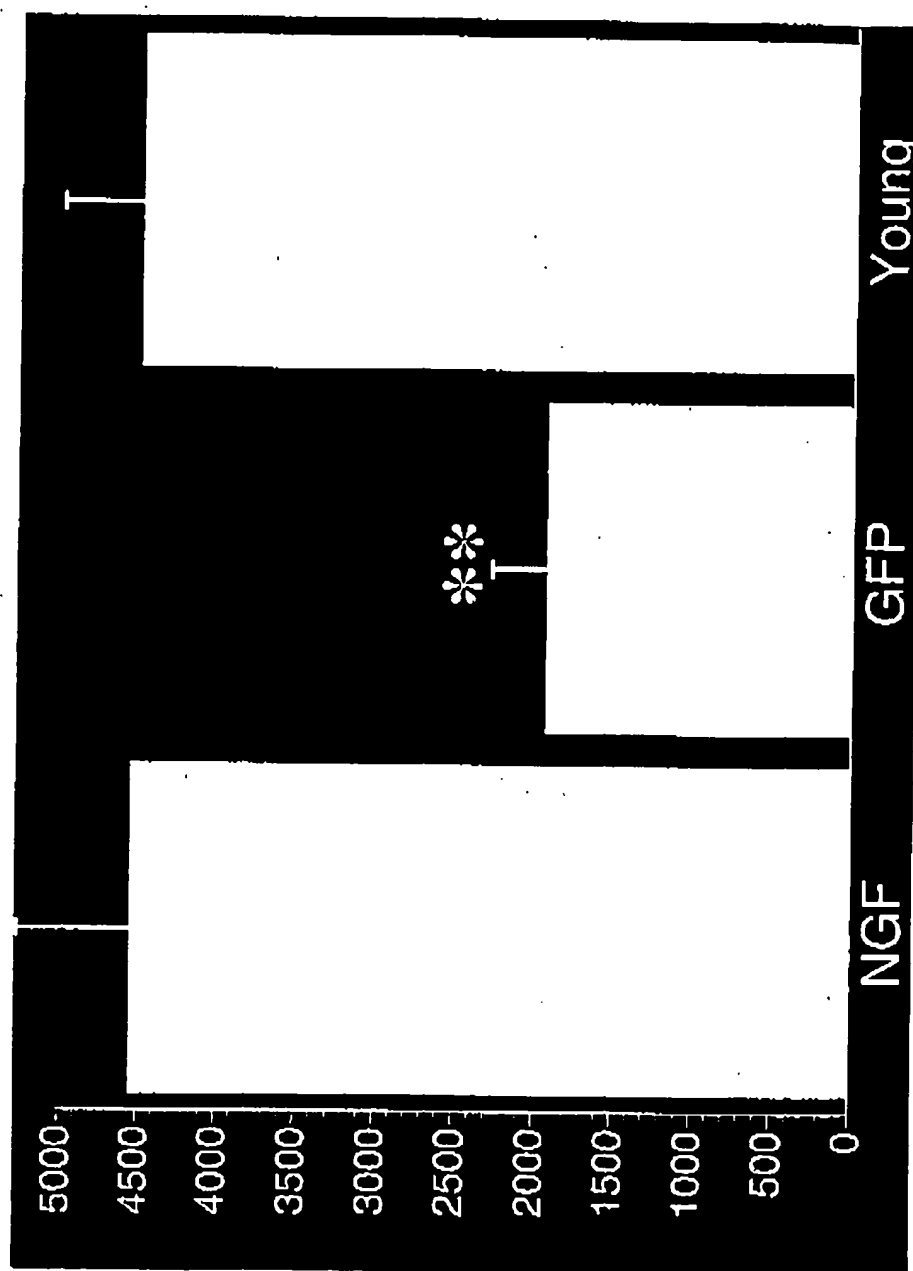
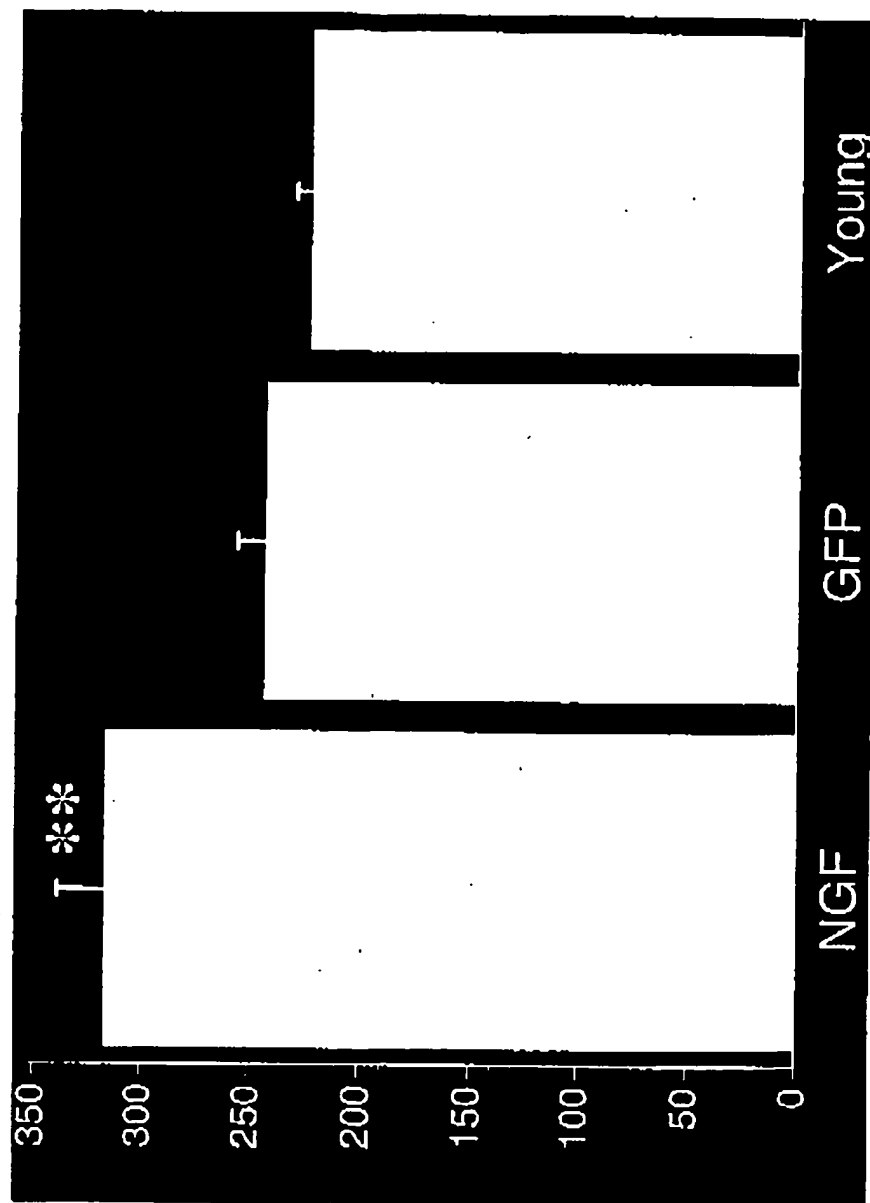


FIGURE 5

Size of p75 labeled Neurons in the Nucleus Basalis



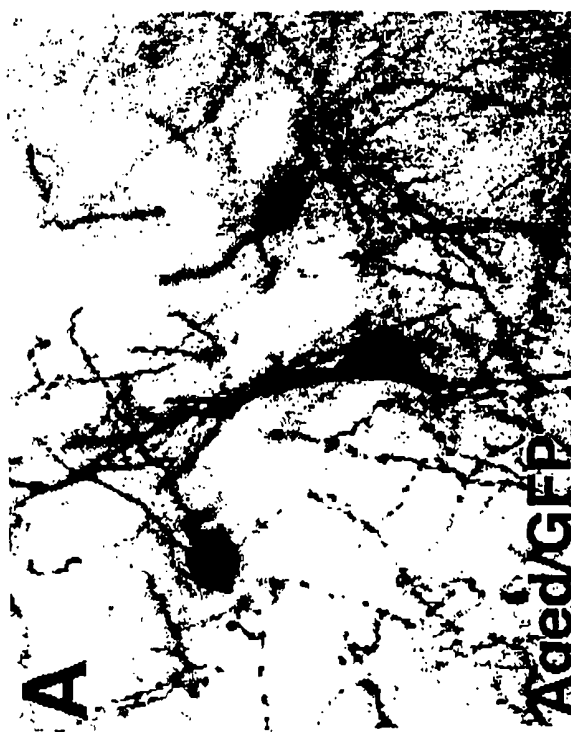
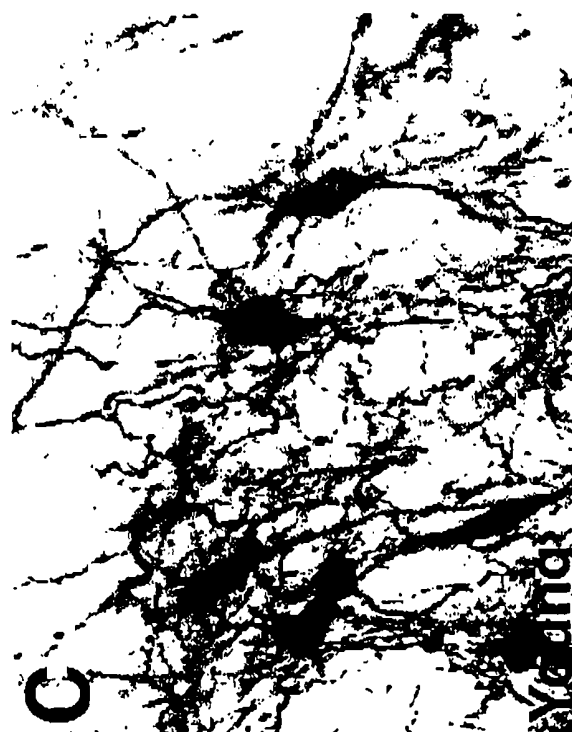
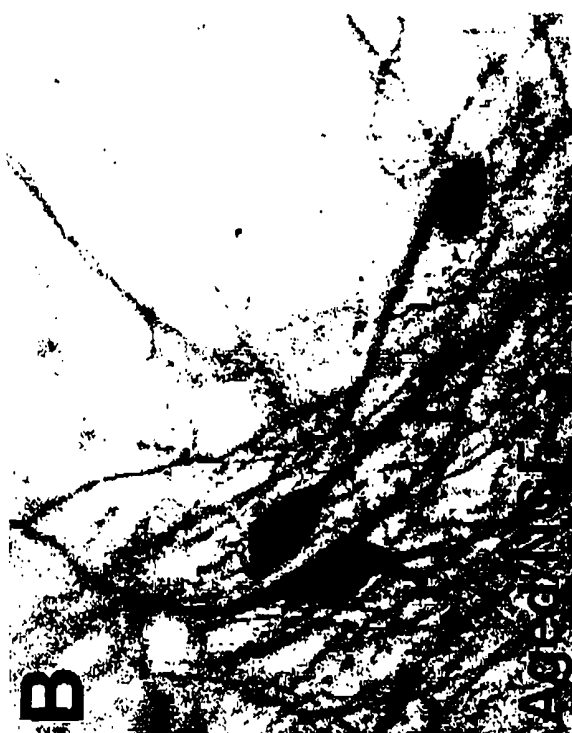
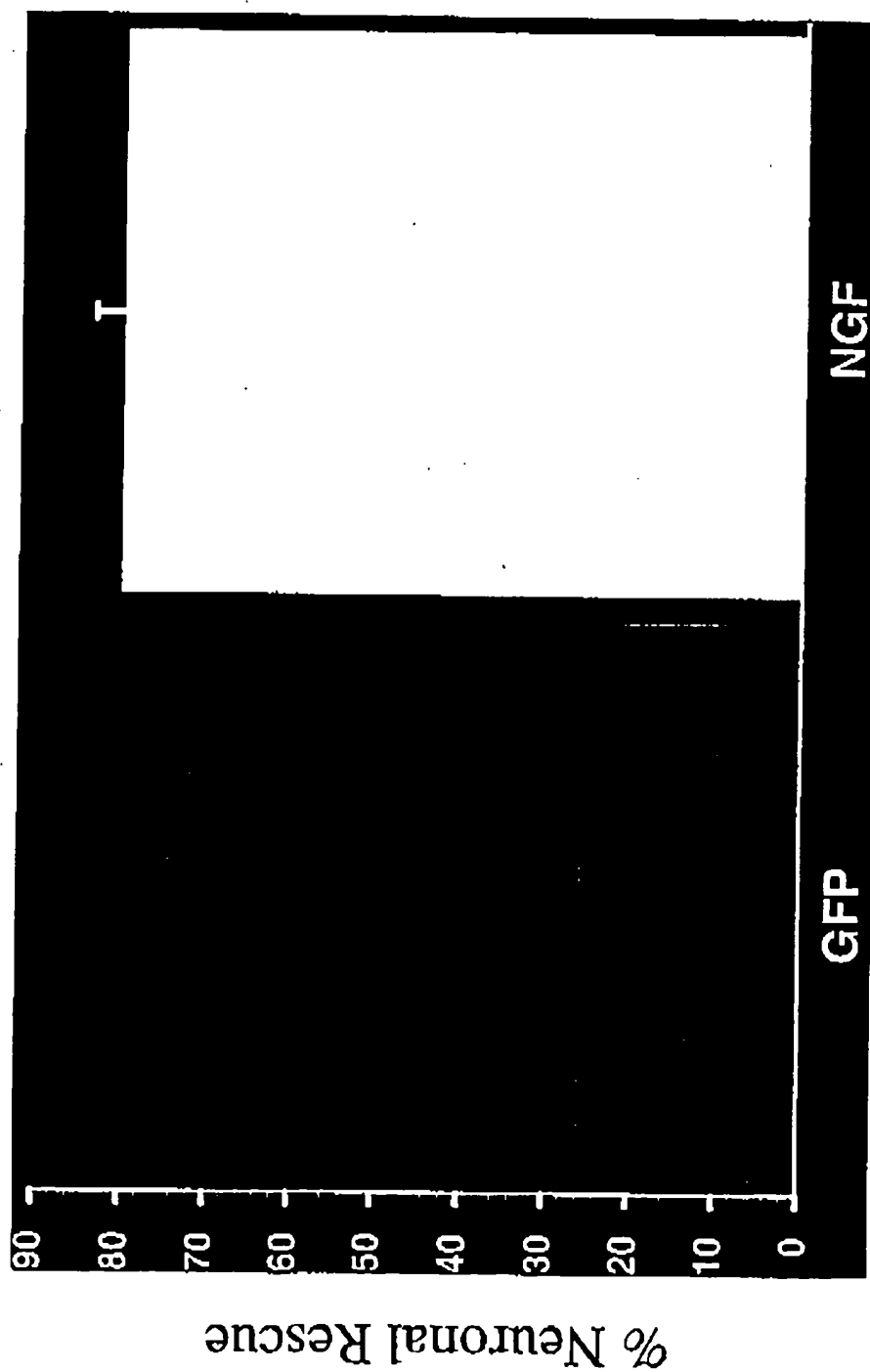


FIGURE 6

FIGURE 7

Cholinergic Neuronal Rescue



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The Immunogenicity of Intracerebral Virus Infection Depends on Anatomical Site

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The brain parenchyma affords immune privilege to tissue grafts, but it is not known whether the same is true for intracerebral viral infections. Using stereotactically guided microinjection, we have confined infection with influenza virus A/NT/60/68 to either the brain parenchyma or the cerebrospinal fluid (CSF). A/NT/60/68 infection in the CSF elicited a comparable immune response to intranasal infection, with the production of antiviral serum antibody, priming of antiviral cytotoxic T-cell precursors, and an antiviral proliferative response in the draining lymph nodes. The response to virus in the CSF was detectable sooner after inoculation than the response to intranasal virus and also involved a prolonged production of virus-specific immunoglobulin A in the CSF. In contrast, there was no detectable immune response to virus infection in the brain parenchyma by any of the parameters measured for at least 10 days after inoculation. Over the next 80 days, 46% of the mice given parenchymal virus developed low-level immune responses that did not involve CSF antibody production, while the remaining 54% had no detectable response at any time. Thus, a virus infection confined to the parenchymal substance of the brain primed the immune system inefficiently or not at all.

An immunological difference between the brain and other anatomical sites was first demonstrated by intracerebral transplantation (3). Allogeneic skin or neural grafts that are rejected in conventional extracerebral sites survive in the brain, although the rejection of an intracerebral graft can be triggered by rejection of an equivalent extracerebral graft. Thus, there is a lack of immune priming by intracerebral grafts rather than a resistance to primed immune effectors. There are regional differences in the degree of intracerebral immune privilege; high degrees of major histocompatibility complex mismatch between donor and recipient lead to a chronic, low-grade rejection of grafts in the cerebral ventricles but not of those in the brain parenchyma (22).

Intracerebral immune privilege is not due to a lack of lymphatic drainage from the brain (6). Despite the absence of specialized lymphatic vessels, there is an efficient functional drainage from the cerebrospinal fluid (CSF) to the deep cervical lymph nodes via the nasal submucosa (2). Extracellular fluid from the brain parenchyma also reaches the nasal submucosal lymphatics, either through the CSF or along perivascular sheaths (5). Consistent with this lymphatic drainage, intracerebral inoculation gives a greater serum antibody response than extracerebral inoculation for a variety of inert antigens, including a bacterial toxin (17), xenogenic erythrocytes (17, 18, 28), and xenogenic albumin (9, 11, 18). Xenogenic albumin in the brain parenchyma elicits a delayed and diminished immune response compared to the same antigen in the CSF but still a greater response than in extracerebral sites (9).

However, it is difficult to extrapolate from proteins, erythrocytes, or tissue grafts to infectious pathogens; differences in the size, availability for degradation, and capacity for cell bind-

ing of antigens are all important considerations in their immunogenicity. Of particular relevance to immune evasion by intracerebral viruses is the lack of resident dendritic cells in the brain parenchyma (12), since they play a key role in initiating immune responses to cell-associated antigens in other organs (23). Many common viruses can infect the central nervous system and cause disease, but the importance of the site of intracerebral infection to any immune response elicited remains unknown.

Influenza virus, apart from being a common respiratory pathogen, has been associated epidemiologically with neuropsychiatric disease in humans (19, 29), and the avian influenza virus strains remain an ever-present potential source of new neurovirulent epidemics (14, 27). Nonneurovirulent influenza viruses such as A/NT/60/68 infect cells in the mouse brain and produce viral proteins but undergo only a single abortive replication cycle (21) and thus remain confined to the site of inoculation (7). They thus provide a suitable tool with which to explore regional differences in the immunogenicity of intracerebral virus infections.

Large-volume intracerebral inoculations are not contained within a precise anatomical site and leak out of the brain parenchyma into the CSF and bloodstream (4, 16). Such inoculations may force antigens into extracerebral sites by pressure-dependent routes that do not contribute to normal lymphatic drainage. We have used stereotactic guidance and an injection volume of 0.5 μ l to confine infection with influenza virus A/NT/60/68 to either the lateral cerebral ventricle (CSF) or the anterior caudoputamen (brain parenchyma). Thus, we have been able to determine how the intracerebral location of a virus infection may influence its capacity to avoid immune priming.

MATERIALS AND METHODS

Mice. C57BL/10 mice were bred at Harlan U.K. Ltd. (Bicester, United Kingdom) and kept under standard Home Office-approved conditions at the Biomedical Services Unit, John Radcliffe Hospital, Oxford, United Kingdom. Intracerebral injections were performed on 6- to 10-week-old mice, matched for age and sex within each experiment. There was no difference in experimental outcome between males and females or across the age range used.

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Virus. Influenza virus A/NT/60/68 was obtained from A. R. Douglas (National Institute for Medical Research, London, United Kingdom) and was grown in 10- to 12-day-old embryonated hen eggs. The virus titer in infectious allantoic fluid was measured by agglutination of chicken erythrocytes and by plaque assay on MDCK cells (1). The same stock allantoic fluid was used for all experiments, with aliquots kept at -80°C and thawed only once before being discarded. The standard 0.5- μl inoculum of neat infectious allantoic fluid contained 5 hemagglutination units (HAU), 5×10^4 PFU, and 5×10^6 electron microscopic viral particles.

Virus administration. Intracerebral injections were performed with the mice immobilized in a stereotaxic frame with the tooth bar at a level 3 mm below the interaural line. The injection coordinates relative to bregma, confirmed by histological inspection, were posterior 0.5 mm, lateral 0.8 mm, and depth 1.7 mm for ventricular injections and anterior 1.0 mm, lateral 2.25 mm, and depth 2.5 mm for parenchymal injections. Mice were anesthetized for the procedure with Hypnorm (fentanyl/flunitrazepam; Janssen Pharmaceuticals, Warrage, United Kingdom) and Hypnovel (midazolam; Roche, Welwyn Garden City, United Kingdom). A hole was made in the skull by using a dentist's drill, and the virus suspension was injected slowly by using a 26S-gauge positive-displacement Hamilton syringe. The skin was sutured after a slow withdrawal of the needle, and all mice made an uneventful recovery. Intracerebral A/NT/60/68 infection in older mice was not associated with significant clinical illness. Intranasal influenza virus was administered under brief ether anesthesia in a volume of 30 μl .

Antibody assays. Serum and CSF anti-A/NT/60/68 antibody titers were determined by enzyme-linked immunosorbent assay (ELISA). Influenza virus was concentrated from infectious allantoic fluid by sucrose density gradient centrifugation (1) and coated overnight (400 ng/well) at 4°C onto 96-well Nunc Polysorb immunoplates (Life Technologies, Paisley, United Kingdom) in sodium bicarbonate (50 mM, pH 9.7)-casein (0.025%) buffer. Coated plates were washed four times with 0.05% Tween in double-distilled H_2O , and nonspecific protein binding was blocked by incubation for 20 min with 2% bovine serum albumin in phosphate-buffered saline-Tween (0.05%)-EDTA (1 mM). After two further washes, duplicate fivefold serum dilutions (100 μl /well) were incubated for 1 h at room temperature followed by five washes. All serum dilutions started at 1/30 and all CSF dilutions started at 1/200 unless otherwise stated. The secondary antibody conjugate was then incubated for 1 h at room temperature followed by a further five washes. The secondary antibodies used were as follows: total immunoglobulin G (IgG), horseradish peroxidase (HRP)-coupled goat anti-mouse Fcy (Sigma Chemical Co., Poole, United Kingdom); IgM, HRP-coupled goat anti-mouse Fcy (Sigma); and IgA, HRP-coupled goat anti-mouse Fcy (Sigma). α -Phenylenediamine dihydrochloride (1 mg/ml; Sigma) in citrate-phosphate buffer (0.1 M, pH 5.5) was used as the HRP substrate, the reaction was terminated with 3 M HCl, and the optical density (OD) was read at 492 nm. Standard immune and naive sera were included on each plate to allow comparison on a plot of OD versus \log_{10} (concentration). Titers were calculated relative to the immune standard for an equivalent OD reading, with this standard assigned an arbitrary number of titer units for each immunoglobulin class, chosen such that the background OD with 1/30 naive mouse serum was equivalent to a titer of 1 U. \log_{10} titers were compared statistically by paired t test.

Neutralizing antiviral antibodies were measured by hemagglutination inhibition (HAI) assay (1). Twofold triplicate serum dilutions were made from an initial 1/5 concentration and were incubated for 30 min at room temperature with 5 HAU of A/NT/60/68 virus. Chicken erythrocytes (Serotec, Kidlington, United Kingdom) were then added (0.5%, final concentration), and the presence or absence of agglutination was scored after a further 30 min. The HAI titer was taken as the reciprocal of the last dilution inhibiting erythrocyte agglutination by the virus in at least two of three wells.

Cytotoxic T-lymphocyte (CTL) stimulation. Spleens were aseptically removed, pooled from pairs of mice, and disrupted into single-cell suspensions. Naive syngeneic feeder spleen cells were incubated for 1 h at 37°C with 200 HAU of influenza virus A/NT/60/68 per 10^6 cells in RPMI (Life Technologies), irradiated (20 Gy), and washed twice in RPMI supplemented with 50 μM 2-mercaptoethanol, 60 μg of penicillin per ml, 100 μg of streptomycin per ml, 2 mM glutamine, and 10% fetal calf serum (FCS; Gibco, Paisley, United Kingdom) (complete medium). Responder spleen cells (10^5 /ml) and feeder spleen cells (3×10^5 /ml) were cultured for 5 days at 37°C with 5% CO_2 in 15 ml of complete medium before testing of cytotoxicity (24).

CTL assay. EL-4 target cells ($H-2^d$) were labelled with ^{51}Cr (100 μCi ; Amersham International, Amersham, United Kingdom) for 1 h at 37°C , washed once, and then incubated with medium alone, pulsed with the $H-2D^b$ -restricted immunodominant influenza virus nucleoprotein peptide (1 μM ASNENMDAM, kindly provided by N. Groome, Oxford Brookes University, Oxford, United Kingdom) (25), or infected with A/NT/60/68 virus (500 HAU/ 10^6 cells). After 1 h at 37°C , the targets were washed twice in complete medium and incubated (10^5 EL-4 cells/well) with effector cells for 4 h before harvesting of supernatants for scintillation counting. Targets with effectors were measured in duplicate, and target controls with Triton or medium alone were measured in quadruplicate. The percent specific lysis was calculated as $100 \times (\text{release by CTL} - \text{release by targets alone})/(\text{release by Triton} - \text{release by targets alone})$. The ^{51}Cr released with medium alone was 5 to 15% of that released by Triton.

Proliferation assay. Lymph nodes were aseptically removed, pooled from pairs of mice, and disrupted into single-cell suspensions. Lymph node cells (2×10^5)

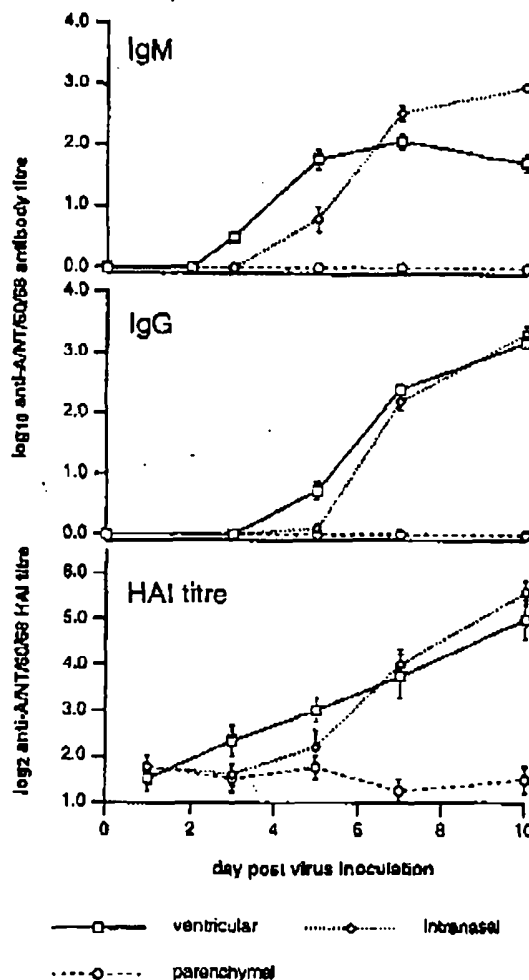


FIG. 1. Early serum anti-A/NT/60/68 antibody responses after intranasal, parenchymal, or ventricular virus inoculation. Intranasal or ventricular but not parenchymal virus inoculations led to the production of specific serum antibody. Means and standard errors of 12 mice per time point from four trials are shown for IgG and IgM titers and for 6 mice per time point from two trials for HAI titers. \log_2 (titer) = 1.6 is the lower limit of HAI assay sensitivity.

were mixed with irradiated syngeneic feeder spleen cells (5×10^4) in quadruplicate 150- μl cultures. Feeder cells were either uninfected, infected with influenza virus A/NT/60/68 as for CTL stimulation, or mixed with phytohemagglutinin (PHA) at a final concentration of 10 $\mu\text{g}/\text{ml}$ (Sigma). The response to PHA gave an indication of the total number of potentially responsive cells; 10 $\mu\text{g}/\text{ml}$ elicits an optimal mitogenic response in this assay system. Culture conditions were as for CTL stimulation (above) except that 1% naive homologous mouse serum was used in place of 10% FCS. After 3 days, 1 μCi of [^3H]thymidine (Amersham International) was added to each well, and the cells were harvested for scintillation counting of incorporated label 18 h later.

Immunohistochemistry. Brains were dissected free of the cranium and outer meninges and quickly frozen in liquid nitrogen. Cryostat sections (7 to 10 μm) were air dried and fixed in acetone for 10 min before staining. All subsequent steps were carried out at room temperature. The sections were preincubated in 10% goat serum and incubated for 1 h with an anti-influenza virus ribonucleoprotein rabbit serum (20), kindly provided by C. Scholtissek (Department of Virology, Justus Liebig University, Giessen, Germany). An HRP-conjugated goat anti-rabbit serum (Vector Laboratories, Peterborough, United Kingdom) was used as the secondary antibody. The sections were washed three times in phosphate-buffered saline with 1% FCS after each incubation. Endogenous

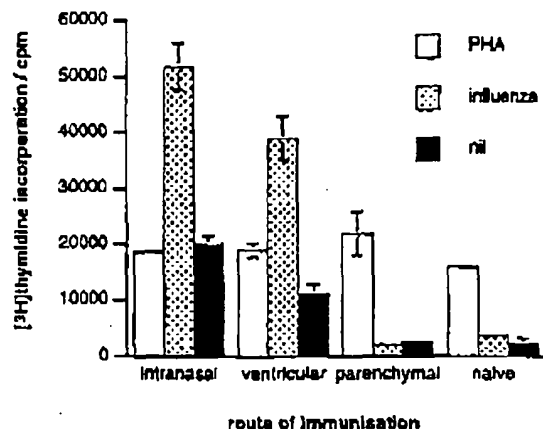


FIG. 2. Proliferative response of cells from the draining lymph nodes 10 days after intranasal, parenchymal, or ventricular virus inoculation. Deep cervical lymph nodes were taken from mice given parenchymal and ventricular virus, mediastinal lymph nodes were taken from mice given intranasal virus, and both sets of lymph nodes (pooled) were taken from naive mice. Cells were pooled from pairs of mice, and each bar shows the mean and standard error of three pairs.

peroxidase activity was blocked with 0.3% hydrogen peroxide in methanol after incubation with the primary antibody. Diaminobenzidine (Sigma) was used as the HRP substrate, sections were counterstained with hematoxylin (Sigma), and slides were mounted with DPX (Merck Ltd., Poole, United Kingdom).

RESULTS

Early antibody response to virus in different sites. Mice were infected with 5 HAU of influenza virus A/NT/60/68 in either the left lateral cerebral ventricle (CSF), the left anterior caudoputamen (brain parenchyma), or the lung. The efficacy of immune priming by each route was determined over the following 10 days by ELISA measurement of total antiviral IgG and IgM and HAI assay of neutralizing antiviral antibody (Fig. 1). Ten days after intranasal or ventricular A/NT/60/68 infection, virus-specific IgG was detectable at a serum dilution of $1/10^3$; 10 days after parenchymal infection, virus-specific IgG was undetectable at a serum dilution of $1/2$.

Early cellular response to virus in different sites. The presence of a virus-specific immune response in the draining lymph nodes was determined 10 days after inoculation by proliferation assay. Virus-specific proliferation was evident with intranasal or ventricular but not with parenchymal virus infections, despite equivalent responses to PHA stimulation (Fig. 2). The high spontaneous proliferation rates seen with mice given intranasal or ventricular virus were probably due to the continued division of cells activated in vivo and to the presence in the responder population of cells presenting viral antigens acquired in vivo. This proliferation provided a further indication of immune activation.

The same mice were tested for the presence of primed CTL precursors 10 days after virus inoculation by restimulating spleen cells in vitro with A/NT/60/68-infected feeder cells prior to cytotoxicity assay (Fig. 3). These results again correlated with the serum antibody titers; virus-specific CTL were primed by intranasal or ventricular infections but not by parenchymal infection. Virus-specific CTL precursors were also detected in the deep cervical lymph nodes of mice given ventricular but not parenchymal A/NT/60/68 (not shown). In six separate trials, there was a consistent agreement between the different immu-

nological parameters; intranasal or ventricular virus always induced specific antibody in the serum, a proliferative response in the lymph nodes, and CTL precursors in the spleen, whereas parenchymal virus failed to induce a response by any of these criteria.

Timing of the immune response to ventricular virus infection. Although ventricular virus infection did not elicit a greater antibody response than intranasal infection, specific serum IgG and IgM were both detectable sooner after virus inoculation into the CSF (Fig. 1). The antiviral antibody response to ventricular influenza virus infection exceeded that to intranasal infection at day 3 ($P < 0.0001$ for IgM) and day 5 ($P < 0.001$ for IgM and $P < 0.001$ for IgG) after virus inoculation.

To determine whether the sensitization of antigen-specific cells in the draining lymph nodes was also more rapid after infection in the CSF, the proliferative responses to ventricular and intranasal virus infections were followed with time after inoculation (Fig. 4). The response to ventricular virus reached high levels by day 3, 2 days earlier than the response to intra-

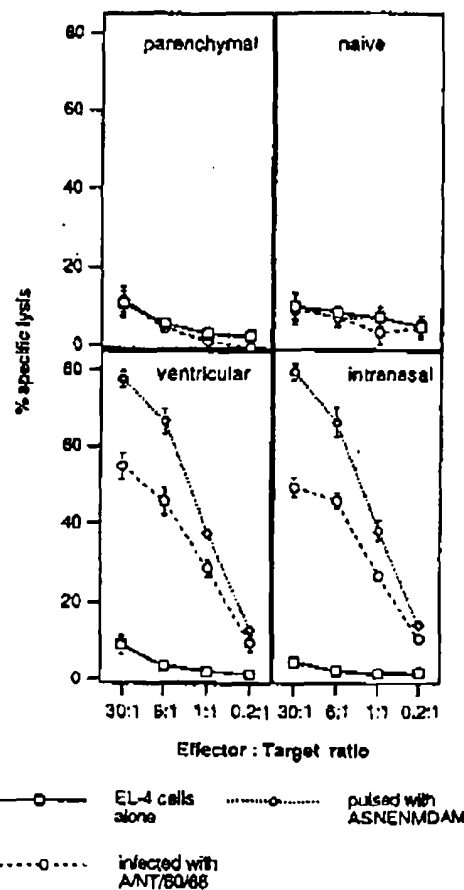


FIG. 3. Cytotoxic activity of restimulated spleen cells 10 days after intranasal, parenchymal, or ventricular virus inoculation. Spleen cells pooled from pairs of mice were restimulated in vitro as described in Materials and Methods before testing of cytotoxicity against EL-4 ($H-2^d$) target cells. Means and standard errors of three pairs of mice are shown in each case. ASNENMDAM is the $H-2D^b$ -restricted immunodominant influenza virus A/NT/60/68 nucleoprotein epitope.

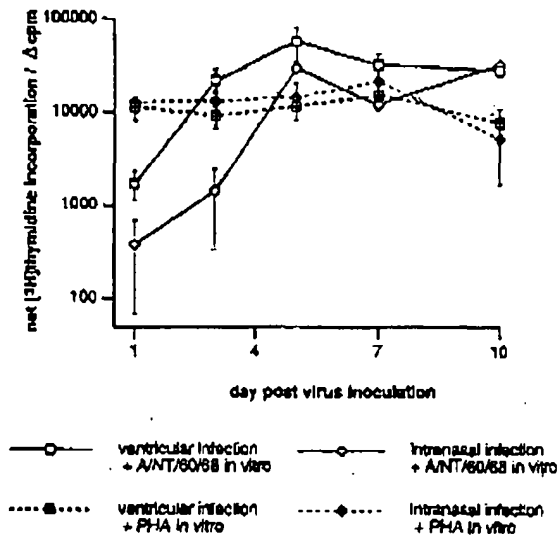


FIG. 4. Time course of proliferative responses from the draining lymph nodes after intranasal or ventricular virus inoculation. Means and standard errors of three trials are shown, each trial including cells pooled from a pair of mice for each time point and for each route of immunization. Δcpm = cpm with the stimulus indicated - cpm with unpulsed feeders. The $[\text{H}]$ thymidine incorporation with unpulsed feeders in these trials did not exceed 1,000 cpm at days 1 to 3, 5,000 cpm at days 5 to 7, or 10,000 cpm at day 10. The response to PHA provided an indicator of the total number of potentially responsive cells in the assay regardless of antigen specificity. For day 1 ventricular and day 1 to 3 intranasal immunizations, the proliferative response with A/NT/60/68-infected feeders was less than three times the response with uninfected feeders and was not considered to be significant. The anti-influenza virus response after parenchymal virus infection did not exceed Δcpm = 500 at any time point (not shown).

nasal virus, corresponding to the earlier onset of serum antibody production after ventricular virus inoculation. Primed virus-specific splenic CTL precursors, however, were detectable at the same time after ventricular or intranasal immunizations (Fig. 5).

Late immune response to intracerebral virus. Immunohistochemistry showed that there was widespread infection of the ependymal cells 1 day after ventricular virus inoculation, but after 10 days, considerable destruction of the ependymal cells had taken place and antiviral staining was minimal (Fig. 6). Virus-infected cells were visible at both 1 and 10 days after parenchymal virus inoculation (Fig. 6) but not in either site after 1 month, when the viral nucleoprotein gene was also undetectable by PCR amplification from total brain cDNA (not shown). Since the nonreplicating virus was apparently lost from the brain by 1 month after inoculation, virus-specific serum antibody was measured by ELISA over 3 months to detect delayed immune responses. Spleen cells from the same mice were restimulated in vitro 3 months after inoculation to detect late CTL priming (Fig. 7). In 7 of 13 (54%) mice given parenchymal virus, there was no detectable antibody at any time. In the remaining 6 (46%) of the 13 mice no antibody was detectable after 10 days but low levels were found from 1 to 3 months, typically maximal at 1 month followed by a slow decline. The presence of primed CTL precursors in the spleen 3 months after parenchymal virus inoculation correlated with the presence of specific serum IgG; mice either had low levels of both or were negative for both. No mice given parenchymal virus had detectable anti-A/NT/60/68 HAI antibody at any time (not shown).

Virus-specific antibody in the CSF. Ventricular infection with influenza virus A/NT/60/68, as with large-volume intracerebral inoculations of other viruses (8, 10), led to a local production of virus-specific immunoglobulin in the CSF (Table 1). Low levels of A/NT/60/68-specific IgG were present in the CSF 10 days after both ventricular and intranasal immunizations, with similar CSF/serum specific IgG ratios, suggesting that the IgG in the CSF was largely derived from that in the serum. But whereas mice given intranasal virus had moderate serum antiviral IgA levels and undetectable CSF levels, mice given ventricular virus had low serum antiviral IgA levels and high CSF levels, consistent with a local synthesis of virus-specific IgA in the CSF. Serum antiviral IgA reached higher levels 90 days after ventricular virus inoculation, accompanied by some diminution in CSF levels. Antiviral IgG or IgA was not detected in the CSF of mice given parenchymal A/NT/60/68 at any time.

DISCUSSION

Viral immunogenicity in the CSF and the brain parenchyma. Large-volume (5- or 30- μl) virus inoculations into the brain parenchyma elicited antibody, proliferation, and cytotoxicity responses equivalent to those elicited by ventricular inoculations (not shown), consistent with a leakage of virus from

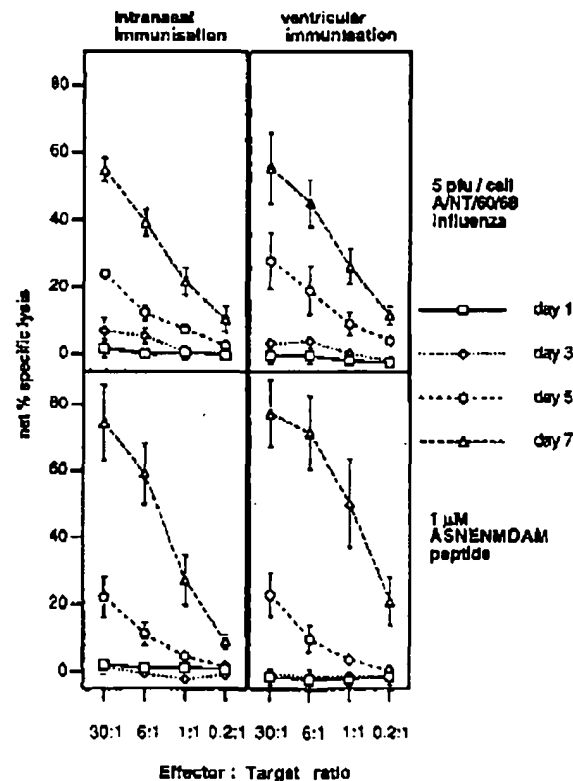


FIG. 5. Cytotoxic activity of restimulated spleen cells with time after intranasal or ventricular virus inoculation. Means and standard errors of three trials are shown, each trial including cells from a pooled pair of mice for each time point and for each route of immunization. Net % specific lysis = % specific lysis of indicated target - % specific lysis of unpulsed targets. Specific lysis of EL-4 cells alone did not exceed 15% at any time point. Net specific lysis after parenchymal virus infection did not exceed 4% at any time point (not shown).



FIG. 6. Intracerebral virus infection 1 and 10 days after parenchymal or ventricular virus inoculation. Anti-influenza virus ribonucleoprotein staining is shown, either 1 day (A and C) or 10 days (B and D) after inoculation of 5 HAU of A/NT/60/68 virus into either the anterior caudoputamen (A and B) or the lateral cerebral ventricle (C and D). Arrows indicate examples of infected cells. After parenchymal virus inoculation, infected intracerebral cells were seen after both 1 day (A) and 10 days (B). After ventricular virus inoculation, almost all the ependymal cells had been infected after 1 day (C), but after 10 days, the virus-infected ependymal lining had largely disappeared. The bar in panel D represents 500 μ m for each panel.

the parenchyma into the CSF. Small-volume (0.5- μ l) virus inoculations remained confined to the site of inoculation and showed a clear difference in immunogenicity between the lateral cerebral ventricle, where infection primed the immune system to a degree comparable with intranasal immunization, and the brain parenchyma, where infection led only to inconsistent, delayed, low-level responses. Apart from the relative rapidity of the response to ventricular virus infection, there were surprisingly few differences between the immune responses to virus in the CSF and to virus in the lung. The local intracerebral immunoglobulin response to influenza virus infection involved IgA production in the CSF (Table 1), analogous to the mucosal IgA response seen with intranasal virus infection (13).

The fate of influenza virus antigens in the CSF. The deep cervical lymph nodes are the main site of an immune response to antigens in the intracranial CSF (11, 15). Although antigens injected intracerebrally have also been associated with immune responses being initiated in the spleen (26, 28), without the use of small-volume and hence low-pressure injections, direct leakage into the bloodstream by nonphysiological routes cannot be ruled out (4, 16). Small-volume inoculations of influenza virus

A/NT/60/68 into the CSF led first to the sensitization of virus-specific cells in the draining lymph nodes (Fig. 4) and then to the appearance of virus-specific antibody in the serum (Fig. 1) and CTL precursors in the spleen (Fig. 5). This was consistent with a primary role for the cervical lymph nodes in the immune response. As with Sindbis virus encephalitis (26), synthesis of virus-specific antibody in the CSF was a relatively late event; A/NT/60/68-specific antibody was not detectable in the CSF 7 days after ventricular virus inoculation (not shown), when specific antibody was abundant in the serum (Fig. 1).

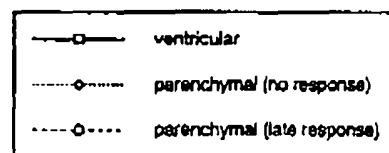
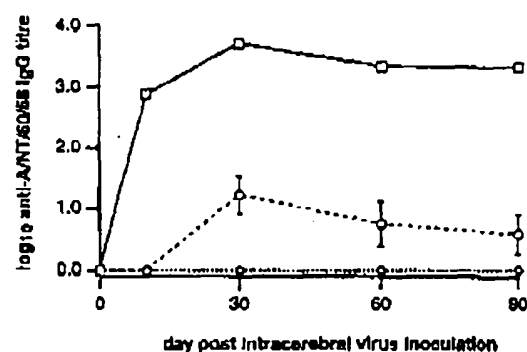


FIG. 7. Late immune response after parenchymal or ventricular virus inoculation. The upper diagram shows A/NT/60/68-specific serum IgG responses over 3 months following virus inoculation, with means and standard errors of six to seven mice per arm pooled from two trials. The mice given parenchymal virus are shown as two separate groups, those which developed late antibody and those which did not. The lower diagram shows the cytotoxic activity of spleen cells from the mice in one representative trial, restimulated *in vitro* with A/NT/60/68-infected syngeneic spleen cells 3 months after intracerebral virus inoculation. Net % specific lysis = % specific lysis of indicated target - % specific lysis of EL-4 cells alone. The specific lysis of EL-4 cells alone did not exceed 15%. Spleen cells from each mouse were restimulated and tested separately; means and standard errors of three mice in each arm are shown.

TABLE 1. Comparison of serum and CSF A/NT/60/68-specific antibody titers at 10 and 90 days after virus inoculation

Route of immunization	Source of antibody	Titer*			
		Anti-A/NT/60/68 IgG		Anti-A/NT/60/68 IgA	
		Day 10	Day 90	Day 10	Day 90
Ventricular	Serum	3.32 \pm 0.08	3.47 \pm 0.15	0.68 \pm 0.14	2.69 \pm 0.10
	CSF	1.49 \pm 0.22	1.91 \pm 0.08	2.63 \pm 0.19	1.96 \pm 0.19
Intranasal	Serum	3.08 \pm 0.19	ND	1.96 \pm 0.13	ND
	CSF	1.41 \pm 0.26	ND	0.00 \pm 0.00	ND
Parenchymal	Serum	0.00 \pm 0.00	0.48 \pm 0.23	0.00 \pm 0.00	0.07 \pm 0.07
	CSF	0.00 \pm 0.00	0.00 \pm 0.00	0.00 \pm 0.00	0.00 \pm 0.00

* Mean \pm standard error log₁₀ anti-A/NT/60/68 antibody titer from 12 to 15 mice. For each route of immunization, the same mice were used for serum and CSF titers. The results for day 90 parenchymal inoculation were pooled from all such mice, the majority of which had no detectable antiviral antibody. ND, not done.

However, we were unable to demonstrate directly viral antigens in the deep cervical lymph nodes after inoculation into the CSF. The viral nucleoprotein gene was undetectable by PCR amplification from cervical lymph node cDNA 1 to 3 days after ventricular virus inoculation, despite being readily amplified from whole brain cDNA at the same time (not shown). Immunohistochemistry also failed to detect the viral nucleoprotein in the deep cervical lymph nodes (not shown), despite its presence in the brain (Fig. 6). These negative results suggested that the antigen reaching the draining lymph nodes either was only a small proportion of the initial inoculum or was present in a different form to the infected cells in the brain. Indirect evidence of immunogenic viral antigens reaching the draining lymph nodes soon after ventricular inoculation was provided by the antiviral proliferative responses (Fig. 4).

The more rapid response to ventricular than to intranasal virus infection was probably due to viral antigens reaching the draining lymphatics directly from CSF (2), whereas intranasal influenza virus first had to cross the respiratory epithelium. A direct entry into lymphatics, avoiding degradation by tissue macrophages and proteases, could also explain the increased intracerebral immunogenicity of a variety of inert antigens (9, 11, 17, 18, 28). An exact quantitative comparison between intranasal and intracerebral virus infections was not possible, since influenza virus A/NT/60/68 replicated productively in the mouse lung (not shown) but not in the mouse brain (21).

After ventricular or larger-volume virus inoculations, influenza virus-specific proliferation was frequently seen (six of nine pairs of mice tested) with cells from the abdominal para-aortic lymph nodes and less often (two of nine pairs of mice tested) with cells from the mediastinal lymph nodes (not shown). These responses were never present without a response in the deep cervical lymph nodes and suggested that virus in the lateral cerebral ventricle may also be carried by CSF flow to more caudal sites with separate lymphatic drainage pathways.

The fate of influenza virus antigens in the brain parenchyma. The simplest interpretation of the lack of response to parenchymal virus infection was that antigens failed to reach the draining lymph nodes. Protein antigens drain efficiently from the brain parenchyma to the cervical lymph nodes (6) and initiate immune responses (9). Influenza virions, however, bind to the abundant sialic acid on cell surfaces and were probably endocytosed before leaving the extracellular spaces of the brain parenchyma. Without virus-infected dendritic cells reaching the lymph nodes, the intracellular viral antigens remained immunologically unseen.

The late low-grade immune responses to parenchymal influenza virus may have resulted from the eventual breakdown of infected cells (when virus was no longer detectable by immu-

nohistochemistry), with viral protein debris being released into the extracellular spaces. Since a late immune response was detected in fewer than half of the mice tested, such debris may often have been degraded before leaving the brain. Nevertheless, persistent immune evasion by intracerebral viruses, whether natural pathogens or used as vehicles for gene therapy, probably depends upon the infected cells not undergoing apoptosis.

Implications for intracerebral virus infection. Although viruses often replicate in extracerebral sites before reaching the brain, congenital transmission, infection of circulating lymphocytes, and retrograde transport along peripheral neurons are all ways by which viruses may reach the brain without first initiating an immune response. These viruses could then remain in the brain parenchyma without priming the immune system. In addition, viruses are able to vary their gene expression according to the cell type infected, and viral proteins expressed specifically in the brain would be unrecognized by the immune system. When immune privilege does contribute to viral persistence, specific extracerebral immune priming might be one way of eliminating infection from the central nervous system.

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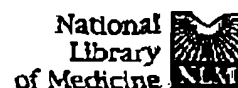
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Viral vectors, tools for gene transfer in the nervous system.

Hermens WT, Verhaagen J.

Graduate School Neurosciences Amsterdam, Netherlands
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Viral vectors are becoming increasingly important tools to investigate the function of neural proteins and to explore the feasibility of gene therapy to treat diseases of the nervous system. This gene transfer technology is based on the virus as a gene delivery vehicle. In contrast to functional analysis of gene products in transgenic mouse, viral vectors can be applied to transfer genes to somatic, post-mitotic cells in developed animals. To date, five viral vector systems are available for gene transfer in the nervous system. These include recombinant and defective herpes viral vectors, adenoviral vectors, adeno-associated viral vectors and lentiviral vectors. In these vectors herpes and adenoviral vectors are the most common in use. To date, one of the main hurdles in applying these two vector systems is the focal immune response that occurs following intraparenchymal infusion. Despite this

limitation, herpes and adenoviral vectors have been used successfully to modify the physiological response to injury in several rodent models of neurodegeneration. The first part of this review is to describe the principles of the generation of viral vector systems currently in use for gene transfer in the nervous system. Secondly, we give an overview of the performance of these vectors following direct infusion in the nervous system and review the results obtained with these vectors in animal models of neurodegeneration and regeneration. The results of these initial studies have provided a framework for future experiments based on gene transfer strategies with viral vectors to study normal physiology and pathology of the nervous system.

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